HHS Public Access

Author manuscript

Clin Infect Dis. Author manuscript; available in PMC 2015 September 14.

Published in final edited form as:

Clin Infect Dis. 2014 September 1; 59(5): 635–642. doi:10.1093/cid/ciu358.

Detection of *Rickettsia rickettsii*, *Rickettsia parkeri*, and *Rickettsia akari* in Skin Biopsy Specimens Using a Multiplex Real-time Polymerase Chain Reaction Assay

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Abstract

Background—*Rickettsia rickettsii*, *Rickettsia parkeri*, and *Rickettsia akari* are the most common causes of spotted fever group rickettsioses indigenous to the United States. Infected patients characteristically present with a maculopapular rash, often accompanied by an inoculation eschar. Skin biopsy specimens are often obtained from these lesions for diagnostic evaluation. However, a species-specific diagnosis is achieved infrequently from pathologic specimens because immunohistochemical stains do not differentiate among the causative agents of spotted fever group rickettsiae, and existing polymerase chain reaction (PCR) assays generally target large gene segments that may be difficult or impossible to obtain from formalin-fixed tissues.

Methods—This work describes the development and evaluation of a multiplex real-time PCR assay for the detection of these 3 *Rickettsia* species from formalin-fixed, paraffin-embedded (FFPE) skin biopsy specimens.

Results—The multiplex PCR assay was specific at discriminating each species from FFPE controls of unrelated bacterial, viral, protozoan, and fungal pathogens that cause skin lesions, as well as other closely related spotted fever group *Rickettsia* species.

Conclusions—This multiplex real-time PCR demonstrates greater sensitivity than nested PCR assays in FFPE tissues and provides an effective method to specifically identify cases of Rocky Mountain spotted fever, rickettsialpox, and *R. parkeri* rickettsiosis by using skin biopsy specimens.

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Disclaimer. The findings and conclusions are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Potential conflicts of interest. All authors: No reported conflicts.
All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Keywords

Rickettsia; real-time PCR; skin biopsies

Spotted fever group *Rickettsia* (SFGR) species are a large and diverse assemblage of obligately intracellular, Gram-negative bacteria that reside in fleas, ticks, and mites. At least 9 named SFGR species are endemic to the United States, including several known pathogens. However, most indigenous spotted fever group (SFG) rickettsioses are caused by *Rickettsia rickettsii*, *Rickettsia akari*, or *Rickettsia parkeri*, the etiologic agents of Rocky Mountain spotted fever (RMSF), rickettsialpox, and *R. parkeri* rickettsiosis, respectively [1]. Each of these infections is characterized by fever and a generalized exanthem, and skin biopsy specimens are often obtained to establish a presumptive diagnosis.

Rickettsia rickettsii is transmitted by several tick species, including *Dermacentor variabilis*, *Dermacentor andersoni*, and *Rhipicephalus sanguineus*. Several hundred to several thousand cases of RMSF are reported annually in the United States, predominantly from the central and southeastern states [2]. RMSF is the most severe SFG rickettsiosis; the case-fatality rate of untreated infections can be >20%. A maculopapular or petechial rash is identified on most patients, but an inoculation eschar is rarely described [3–5]. *Rickettsia akari* is transmitted to humans from the bite of the house mouse mite (*Liponyssoides sanguineus*) [6]. Rickettsialpox is milder than RMSF and is typically associated with an inoculation eschar and a maculopapular rash that may be vesicular. In the United States, rickettsialpox exists as an urban zoonosis, and almost every documented US case has originated from a large metropolitan center [7]. The intentional release of *Bacillus anthracis* as a weapon of bioterrorism in 2001 elevated physician awareness of eschar-associated illnesses, including rickettsialpox [8]. *Rickettsia parkeri* was first identified as a cause of disease in humans in 2004 [9]. The Gulf Coast tick (*Amblyomma maculatum*) is the vector of *R. parkeri* and is distributed throughout much of the southeastern and mid-Atlantic United States [10]. This moderately severe illness shares features with RMSF and rickettsialpox, namely, the occurrence of 1 or more inoculation eschars, and a maculopapular rash, occasionally with vesicular or petechial components [9].

The sympatric distribution of the tick vectors and *Rickettsia* species, and the clinical and histological similarities of 1 or more of the cutaneous manifestations of RMSF, *R. parkeri* rickettsiosis and rickettsialpox (Figures 1 and 2), necessitate the use of advanced methods to confirm and distinguish these infections. In addition, various other viral, bacterial, fungal, or protozoan pathogens may cause eschar or rash lesions that are clinically or histologically similar to those caused by SFGR [10, 11]. Immunohistochemical staining techniques are useful to confirm SFG rickettsioses; however, these assays are not species specific [7,9]. Species-specific identification of the diseases is achieved infrequently from formalin-fixed, paraffin-embedded (FFPE) specimens [12], because relatively large segments of particular gene targets are used conventionally to establish a molecular diagnosis from blood or fresh tissues [13]. Species-specific confirmation from FFPE skin biopsy specimens is particularly challenging because formalin causes nucleic acid fragmentation that characteristically limits the size of successful polymerase chain reaction (PCR) amplicons [12], and skin biopsies

typically provide relatively small amounts of pathogen DNA for molecular analysis. This work was initiated to develop a reliable real-time PCR assay to amplify small but specific DNA fragments of 3 of the most frequently encountered pathogenic SFGR in the United States from FFPE skin biopsy specimens.

MATERIALS AND METHODS

Selection and Preparation of Controls

FFPE control blocks were prepared from Vero E6 cells infected separately with 10 *Rickettsia* species that included (1) recognized pathogens endemic to the United States (*R. rickettsii*, *R. parkeri*, *R. akari*, and *Rickettsia felis*); (2) pathogenic species associated with international travel (*Rickettsia australis*, *Rickettsia africae*, *Rickettsia conorii*, *Rickettsia sibirica*, and *Rickettsia massiliae*), and (3) a *Rickettsia* species of undetermined pathogenicity (*Candidatus* "Rickettsia amblyommii") [14] (Table 1). Additional controls included FFPE blocks containing cells or tissues infected with unrelated viral, bacterial, protozoan, and fungal pathogens that may cause cutaneous lesions, including human herpesviruses 1, 2, and 3, orf virus, *Bacillus anthracis*, *Francisella tularensis*, *Yersinia pestis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Capnocytophaga canimorsus*, *Neisseria meningitidis*, *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, *Burkholderia pseudomallei*, *Leptospira* species, *Salmonella typhi*, *Sporothrix schenckii*, and *Leishmania mexicana mexicana*.

Case-patient specimens for evaluation included 31 FFPE skin biopsy specimens submitted to the Infectious Diseases Pathology Branch at the Centers for Disease Control and Prevention during the years 2001 through 2013 that tested positive for infection with a SFGR species by immunohistochemical (IHC) staining. Among this set of specimens included 16 for which a species-specific diagnosis was available by culture or nested *ompA* gene PCR evaluation of fresh tissue, blood, or another specimen [18]. The remaining 15 samples were selected based on clinical and epidemiological features that were compatible with a presumptive diagnosis of RMSF, *R. parkeri* rickettsiosis, or rickettsialpox (Table 2).

DNA Extraction

Four 16-μm sections were cut from each paraffin block and placed in a microcentrifuge tube. The sections were deparaffinized with xylene and washed twice with absolute ethanol. Tissues were incubated overnight at 56°C in 180 μL Buffer ATL and 20 μL proteinase K (Qiagen, Valencia, California). Extraction of the supernatant was completed using a Qiagen QIAamp DNA Micro Kit and the manufacturer's "Tissues" protocol, with a final elution volume of 50 μL.

Molecular Assays

Primer and probe sequences for real-time PCR are listed in Table 3. For detection of SFGR, real-time PCR primers targeting the citrate synthase gene (*gltA*) were used [19]. The reaction consisted of 12.5 μL of Qiagen QuantiTect Multiplex PCR Master Mix, 0.4 μM of each of the forward and reverse primers, 0.2 μM of the probe, and 2.5 μL of DNA extract in a 25-μL volume. Cycling conditions consisted of 1 cycle of 95°C for 15 minutes, and 45

cycles of 95°C for 60 seconds and 60°C for 60 seconds using an Agilent (Santa Clara, California) Mx3005P real-time thermal cycler.

Primers and probes targeting a hypothetical protein gene of the *R. rickettsii* genome [20] and the *ompB* genes of *R. parkeri* [21] and *R. akari* were multiplexed into a single tube real-time assay. Reactions were comprised of 12.5 μL of Qiagen QuantiTect Multiplex PCR Master Mix, 0.2 μM of each of the forward and reverse primers and probes, and 2.5 μL of DNA extract in a 25-μL volume. Cycling conditions consisted of 1 cycle of 95°C for 15 minutes, and 45 cycles of 94°C for 60 seconds and 60°C for 90 seconds using an Agilent Mx3005P real-time thermal cycler. All PCR reactions included appropriate positive and negative controls, and samples were considered positive if the cycle threshold value was ≤40 for each of the respective targets.

Conventional nested PCR assays to the *ompA* gene and the 17 kDa antigen gene have long been considered the molecular reference standard for SFGR detection and were used in this work as a comparison to the real-time PCR assays. The *ompA* PCR was performed as previously described [18]. Heminested PCR to the 17 kDa antigen gene was performed using primers TZ15 and TZ16 [15] in the primary stage, and a Roche (Indianapolis, Indiana) High Fidelity PCR Master Kit with final primer concentrations of 300 nmol and 2.5 μL DNA extract in a 25-μL reaction volume. Reactions were denatured at 94°C for 2 minutes; then subjected to 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 45 seconds; and a final extension at 72°C for 5 minutes. The heminested stage produced a 218-bp amplicon with primers TZ-FN (5′-TTG TBG GAG TAG GTG TAG) and TZ16, and the reaction used 1 μL of the primary PCR reaction and the same cycling conditions as above, except the total number of cycles was reduced to 30.

Amplicons from the *ompA* and 17 kDa antigen PCR assays were agarose gel-purified using a Qiagen QiaQuick Gel Extraction Kit. Cycle sequencing of the eluates was performed using Quick Start DTCS Master Mix (Beckman Coulter, Indianapolis, Indiana). Reactions were purified using a Qiagen DyeEx 2.0 Spin Kit, concentrated by vacuum centrifugation, suspended in 35 μL of Sample Loading Solution (Beckman Coulter), and placed on a GenomeLab GeXP (Beckman Coulter) for sequencing. Consensus nucleotide sequences were analyzed using CLC Main Workbench (Cambridge, Massachusetts) and the National Center for Biotechnology Information's Basic Local Alignment Search Tool program (http://blast.ncbi.nlm.nih.gov).

RESULTS

The multiplex PCR assay correctly identified all cell controls for *R. rickettsii*, *R. parkeri*, and *R. akari*. No false-positive reactions were detected with any of the infected cell culture controls containing other *Rickettsia* species or other viral, bacterial, fungal, or protozoan pathogens (Table 1). As expected, DNA extracts from *R. massiliae*, *R. felis*, and *R. australis* did not produce amplicons with the *ompA* primer set [16, 17, 22], and DNA extracted from *R. akari* did not produce amplicons with the *ompA* [17] or 17 kDa antigen primer set due to mismatches between the nucleotide sequences.

DNA extracted from FFPE skin biopsy specimens from 6 cases of culture-confirmed or *ompA* nested PCR–confirmed [18] *R. parkeri* infection were tested with the real-time PCR assay; all 6 cases were positive for *R. parkeri* and negative for *R. rickettsii* and *R. akari*. Three confirmed cases of fatal RMSF and 2 confirmed cases of rickettsialpox were positive for their respective targets and negative for the targets of the 2 other *Rickettsia* species. DNA extracts from FFPE biopsies obtained from 5 patients with PCR-confirmed African tick bite fever and from 1 patient with PCR-confirmed infection with *Rickettsia* 364D [23] were positive when tested for the *Rickettsia* citrate synthase gene (*gltA*) [19], but negative for *R. parkeri, R. rickettsii, and R. akari* when tested by the multiplex real-time PCR assay.

Several cases of suspected SFGR infection were also analyzed to determine a causative species. Each of the suspected cases was negative by *ompA* and 17 kDa antigen gene PCR. Based on their geographical origin and clinical characteristics, the evaluation included 10 suspected cases of rickettsialpox from New York City, 4 suspected cases of *R. parkeri* rickettsiosis from Maryland, South Carolina, Texas, and Virginia, and 1 suspected case of fatal RMSF from Mexico. All 15 suspected cases were positive by *gltA* real-time PCR, indicating the presence of rickettsial DNA. The suspected RMSF case was positive for the *R. rickettsii* hypothetical protein target only. All suspected rickettsialpox cases were positive only for the *R. akari ompB* gene in the real-time assay. Of the 4 suspected cases of *R. parkeri* rickettsiosis, only 3 were positive by the real-time assay for the *R. parkeri ompB* gene. The fourth suspected case was instead positive for the *R. akari ompB* gene.

DISCUSSION

This study is the first to utilize a multiplex real-time PCR assay for the detection of *R. rickettsii*, *R. akari*, and *R. parkeri* in FFPE skin biopsy specimens. From culture- and PCR-confirmed cases, *R. parkeri*, *R. rickettsii*, and *R. akari* were successfully identified. We were able to determine a causative species using the real-time PCR assay in biopsies from several suspected cases of IHC-positive SFGR infection. One case of SFG rickettsiosis in a 76-year-old woman from Maryland was initially suspected to be caused by *R. parkeri* and was unexpectedly discovered to be positive for *R. akari*. Accurate identification of the infecting *Rickettsia* species is critically important to establish valid distributions and clinical consequences associated with the SFG rickettsioses [24], and there are possibly many examples of incorrect associations based on nonspecific assays [25].

There are certain limitations to this method. Immunohistochemical analysis of skin biopsy specimens reveals that rickettsial antigens are not uniformly distributed throughout the tissue. Whereas one tissue section may contain abundant antigen and nucleic acid, a tissue section cut from deeper into the FFPE block may have markedly less rickettsial nucleic acid available for analysis [26]. Exposure of the FFPE block to air or prolonged fixation of the tissue specimen in formalin can also affect the quality and quantity of nucleic acid available for amplification [27]. The timing of the biopsy procedure during the course of infection and the interval from administration of an appropriate antibiotic (ie, doxycycline) prior to biopsy may also affect the quantity of amplifiable nucleic acids in the biopsy sample [26]. This multiplex assay confirmed the infecting SFG *Rickettsia* species in 15 case-patients that would not have been known otherwise; nonetheless, infections caused by other *Rickettsia*

species endemic to the United States, such as *Rickettsia* species 364D and *R. felis* [23, 28], and those associated with foreign travel, such as *R. africae* [29], are not specifically identified by this multiplex assay, reflecting a need for development of additional molecular methods to confirm rickettsioses from FFPE skin biopsy specimens.

Formalin fixation of skin biopsy specimens allows for histologic and IHC analysis, but due to cross-linking and fragmentation of nucleic acids, performing PCR on FFPE samples can be difficult due to the large amplicon sizes and nested PCR protocols needed to obtain sufficient sequencing data for *Rickettsia* species differentiation. These are also time-consuming processes. By developing this multiplex real-time PCR assay, a species-specific diagnosis can be obtained in <3 hours, as compared to the ≥8 hours needed to amplify and sequence products obtained by conventional nested PCR.

This multiplex real-time PCR assay was developed to evaluate nucleic acids extracted from FFPE skin biopsy specimens, but it is also possible that these methods can be effectively adapted for use on a variety of other specimen types. Detection of rickettsial DNA has been increasingly reported using swabs of eschars [30–33], and quantitative PCR of swab material collected as late as 2 weeks after the start of antibiotic therapy may provide a confirmatory result in some cases [32]. We have also successfully applied this multiplex assay to obtain species-specific diagnoses from other types of FFPE tissues (unpublished data).

In conclusion, this multiplex real-time PCR establishes a rapid method to distinguish these infections from other infectious causes of eschar- and rash-associated illnesses, and can provide more accurate information on the distribution and clinical characteristics of these rickettsioses by establishing a species-specific diagnosis.

Acknowledgments

We thank Maureen G. Metcalfe for sectioning of FFPE blocks and for review of the manuscript.

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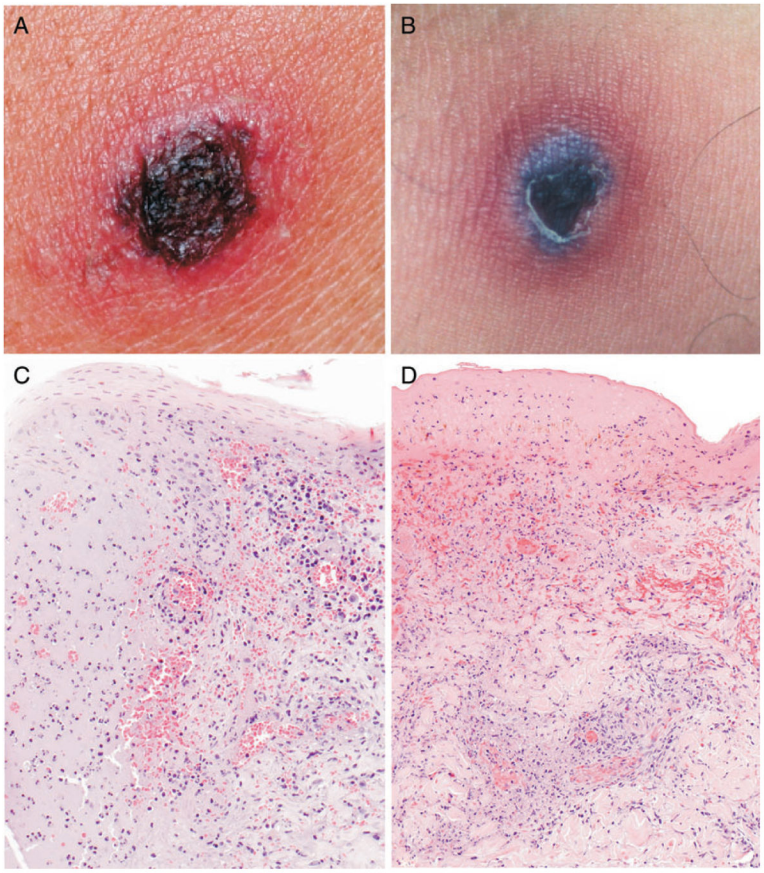

Figure 1.

Clinical and histological resemblance between the inoculation eschar of *Rickettsia parkeri* rickettsiosis (*A* and *C*) and rickettsialpox (*B* and *D*). Clinically, both lesions are characterized by a 0.5- to 1.5-cm necrotic crust surrounded by an erythematous halo (*A* and *B*). Microscopically, these demonstrate indistinguishable features comprising necrosis of the epidermis and upper dermis, hemorrhage, and perivascular neutrophilic and lymphohistiocytic inflammatory cell infiltrates accompanied by occlusive fibrin thrombi (*C* and *D*) (hematoxylin and eosin stain, original magnifications ×25).

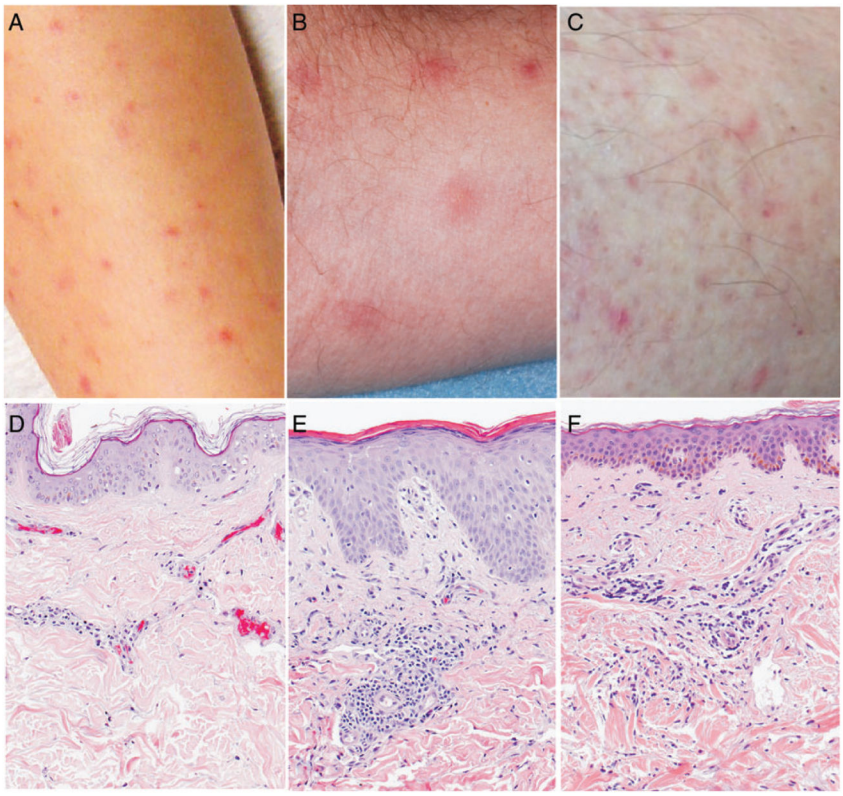

Figure 2.

Clinical and histological similarities among rashes of Rocky Mountain spotted fever (*A* and *D*), *Rickettsia parkeri* rickettsiosis (*B* and *E*), and rickettsialpox (*C* and *F*). Each infection may exhibit an erythematous maculopapular rash (*A*–*C*). The histological features of these rashes are often quite similar and are characterized predominantly by lymphohistiocytic perivascular inflammatory cell infiltrates (*C*–*E*) (hematoxylin and eosin stain, original magnifications ×25).

Table 1

Controls Used in This Study to Assess the Specificity of the Multiplex Polymerase Chain Reaction Assay

	Real-time PCR Assays				Conventional PCR Assays	
Name	**Citrate Synthase (*gltA*) Gene**	***R. rickettsii* Hypothetical Protein Gene**	***R. parkeri ompB* Gene**	***R. akari ompB* Gene**	***ompA* Gene**	**17 kDa Antigen Gene**
R. akari strain Columbia 1	+	−	−	+	_[a]	_[a]
R. parkeri strain Deep Creek	+	−	+	−	+	+
R. australis strain NQTT	+	−	−	−	_[a]	+
R. rickettsii strain AZ-3	+	+	−	−	+	+
R. felis strain LSU	+	−	−	−	_[a]	+
R. conorii strain #7	+	−	−	−	+	+
R. massiliae strain Mtu1 (ATCC VR-1376)	+	−	−	−	_[a]	+
R. africae strain Z9-Hu	+	−	−	−	+	+
R. sibirica strain 246 (ATCC VR-151)	+	−	−	−	+	+
“*R. amblyommii*” strain Darkwater	+	−	−	−	+	+
Bacillus anthracis	−	−	−	−	−	−
Francisella tularensis	−	−	−	−	−	−
Yersinia pestis	−	−	−	−	−	−
Pseudomonas aeruginosa	−	−	−	−	−	−
Sporothrix schenckii	−	−	−	−	−	−
Staphylococcus aureus	−	−	−	−	−	−
Capnocytophaga canimorsus	−	−	−	−	−	−
Neisseria meningitidis	−	−	−	−	−	−

Name	Real-time PCR Assays				Conventional PCR Assays	
	Citrate Synthase (*gltA*) Gene	*R. rickettsii* Hypothetical Protein Gene	*R. parkeri ompB* Gene	*R. akari ompB* Gene	*ompA* Gene	17 kDa Antigen Gene
Ehrlichia chaffeensis	–	–	–	–	–	–
Anaplasma phagocytophilum	–	–	–	–	–	–
Burkholderia pseudomallei	–	–	–	–	–	–
Leptospira species	–	–	–	–	–	–
Salmonella typhi	–	–	–	–	–	–
Leishmania mexicana mexicana	–	–	–	–	–	–
Human herpesvirus 1	–	–	–	–	–	–
Human herpesvirus 2	–	–	–	–	–	–
Human herpesvirus 3	–	–	–	–	–	–
Orf virus	–	–	–	–	–	–

Abbreviation: PCR, polymerase chain reaction.

[a] Expected result based on primer mismatch [15–17].

Table 2

Real-time Polymerase Chain Reaction Evaluation of Formalin-Fixed, Paraffin-Embedded Skin Biopsy Specimens Submitted to the Centers for Disease Control and Prevention for Confirmation of a Spotted Fever Group Rickettsiosis

			Real-time PCR Assays				Conventional Nested PCR Assays	
Disease	**Case Designation**	**Case Status Prior to Real-time PCR Analysis**	**Citrate synthase (*gltA*) Gene**	***R. rickettsii* Hypothetical Protein Gene**	***R. parkeri ompB* Gene**	***R. akari ompB* Gene**	***ompA* Gene**	**17 kDa Antigen Gene**
Rocky	AZ1	Confirmed[a]	+	+	−	−	−	+
Mountain	CR1	Confirmed	+	+	−	−	−	+
spotted fever	MX1	Suspected	+	+	−	−	−	−
	MX2	Confirmed	+	+	−	−	−	−
Rickettsia	AL1	Confirmed	+	−	+	−	+	+
parkeri	GA1	Confirmed	+	−	+	−	+	+
rickettsiosis	MS1	Confirmed	+	−	+	−	+	−
	MS2	Confirmed	+	−	+	−	−	−
	SC1	Confirmed	+	−	+	−	−	−
	SC2	Suspected[b]	+	−	+	−	−	+
	TX1	Confirmed	+	−	+	−	−	−
	TX2	Suspected	+	−	+	−	−	−
	VA1	Suspected	+	−	+	−	−	+
Rickettsialpox	MD1	Suspected[c]	+	−	−	+	−	−
	NY1	Confirmed	+	−	−	+	−	−
	NY2	Suspected	+	−	−	+	−	−

Disease	Case Designation	Case Status Prior to Real-time PCR Analysis	Real-time PCR Assays				Conventional Nested PCR Assays	
			Citrate synthase (*gltA*) Gene	*R. rickettsii* Hypothetical Protein Gene	*R. parkeri ompB* Gene	*R. akari ompB* Gene	*ompA* Gene	17 kDa Antigen Gene
	NY3	Confirmed	+	−	−	+	−	−
	NY4	Suspected	+	−	−	+	−	−
	NY5	Suspected	+	−	−	+	−	−
	NY6	Suspected	+	−	−	+	−	−
	NY7	Suspected	+	−	−	+	−	−
	NY8	Suspected	+	−	−	+	−	−
	NY9	Suspected	+	−	−	+	−	−
	NY10	Suspected	+	−	−	+	−	−
	NY11	Suspected	+	−	−	+	−	−
	NY12	Suspected	+	−	−	+	−	−
African tick bite fever	AL2	Confirmed	+	−	−	−	+	+
	FL1	Confirmed	+	−	−	−	+	+
	PA1	Confirmed	+	−	−	−	+	+
	MD2	Confirmed	+	−	−	−	+	+
Rickettsia 364D rickettsiosis	CA1	Confirmed	+	−	−	−	−	+

Abbreviation: PCR, polymerase chain reaction.

[a] Confirmed independently by culture isolation from blood or fresh tissue or by conventional PCR.

[b] Clinically and epidemiologically compatible illness and biopsy specimen staining positive for a spotted fever group *Rickettsia* species by immunohistochemistry.

[c] Suspected initially to be *R. parkeri* rickettsiosis.

Table 3

Targets and Primer and Probe Sequences Used in This Study

Target	5′ to 3′ Sequence	Reference
Rickettsia species citrate synthase (*gltA*) gene	TCG CAA ATG TTC ACG GTA CTT T TCG TGC ATT TCT TTC CAT TGT G FAM-TGC AAT AGC AAG AAC CGT AGG CTG GAT G-BHQ1	[19]
R. rickettsii hypothetical protein gene	AAA TCA ACG GAA GAG CAA AAC CCC TCC ACT ACC TGC ATC AT CY5-TCC TCT CCA ATC AGC GAT TC-BHQ3	[20]
R. parkeri ompB gene	CAA ATG TTG CAG TTC CTC TAA ATG AAA ACA AAC CGT TAA AAC TAC CG FAM-CGC GAA ATT AAT ACC CTT ATG AGC AGC AGT CGC G-BHQ1	[21]
R. akari ompB gene	GTG GTG CTG TTG CAG GTG G TTG CTC CAC CGA GAG TTA ATG TT HEX-CGG TGC TGG TAA TGC TGC ATT ACA CG-BHQ1	This study